ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2007, p. 4329–4335 Vol. 51, No. 12
0066-4804/07/$08.00+0 doi:10.1128/AAC.00810-07

Molecular Epidemiology and Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa* Isolates from Spanish Hospitals[▽]

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Received 22 June 2007/Returned for modification 30 August 2007/Accepted 3 October 2007

All (236) *Pseudomonas aeruginosa* isolates resistant to imipenem and/or meropenem collected during a multicenter (127-hospital) study in Spain were analyzed. Carbapenem-resistant isolates were found to be more frequently resistant to all β-lactams and non-β-lactam antibiotics than carbapenem-susceptible isolates ($P < 0.001$), and up to 46% of the carbapenem-resistant isolates met the criteria used to define multidrug resistance (MDR). Pulsed-field gel electrophoresis revealed remarkable clonal diversity (165 different clones were identified), and with few exceptions, the levels of intra- and interhospital dissemination of clones were found to be low. Carbapenem resistance was driven mainly by the mutational inactivation of OprD, accompanied or not by the hyperexpression of AmpC or MexAB-OprM. Class B carbapenemases (metallo-β-lactamases [MBLs]) were detected in a single isolate, although interestingly, this isolate belonged to one of the few epidemic clones documented. The MBL-encoding gene (bla_{VIM-2}), along with the aminoglycoside resistance determinants, was transferred to strain PAO1 by electroporation, demonstrating its plasmid location. The class 1 integron harboring bla_{VIM-2} was characterized as well, and two interesting features were revealed: *intI1* was found to be disrupted by a 1.1-kb insertion sequence, and a previously undescribed aminoglycoside acetyltransferase-encoding gene [designated *aac(6′)-32*] preceded bla_{VIM-2}. AAC(6′)-32 showed 80% identity to AAC(6′)-Ib′ and the recently described AAC(6′)-31, and when *aac(6′)-32* was cloned into *Escherichia coli*, it conferred resistance to tobramycin and reduced susceptibility to gentamicin and amikacin. Despite the currently low prevalence of epidemic clones with MDR, active surveillance is needed to detect and prevent the dissemination of these clones, particularly those producing integron- and plasmid-encoded MBLs, given their additional capacity for the intra- and interspecies spread of MDR.

The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality (20, 27). The growing threat of antimicrobial resistance in *P. aeruginosa* lies on one hand in the extraordinary capacity of this microorganism to develop resistance to almost any available antibiotic by the selection of mutations in chromosomal genes and, on the other, in the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenemases (or metallo-β-lactamases [MBLs]) (21).

Among the particularly noteworthy mutation-mediated resistance mechanisms are those leading to the repression or inactivation of the porin OprD, conferring resistance to imipenem and reduced susceptibility to meropenem (7, 36, 42, 50), and those leading to the hyperexpression of the chromosomally encoded cephalosporinase AmpC, conferring resistance to penicillins and cephalosporins (12, 13). Also remarkable, mutations leading to the up-regulation of one of the several efflux pumps encoded in the *P. aeruginosa* genome may confer resistance or reduced susceptibility to multiple agents, including all β-lactams (except imipenem), fluoroquinolones, and aminoglycosides (4, 10, 22, 25, 28, 39). Furthermore, the accumulation of various combinations of these chromosomal mutations can certainly lead to the emergence of MDR (or even pan-antibiotic-resistant) strains, which eventually may be responsible for notable epidemics in the hospital setting (6). The problem of mutation-mediated multidrug resistance is further amplified in chronic respiratory infections due to the high prevalence of hypermutable strains (24, 29).

In addition to the mutation-mediated resistance, the presence of horizontally acquired resistance determinants in *P. aeruginosa* has been increasingly reported over the last decade. Among the certainly noteworthy determinants are those encoding MBLs, particularly IMP and VIM enzymes, which are able to hydrolyze efficiently all β-lactams with the exception of aztreonam (48). Since IMP-1 was first detected in Japan in the early 1990s, MBL-producing strains have been increasingly reported worldwide and have been responsible for large outbreaks in several Asian, European, and American hospitals (15, 18, 23, 30, 31, 45, 48). Genes encoding MBLs are generally located within class 1 integrons, together with those encoding aminoglycoside-modifying enzymes that confer multidrug resistance (19, 26, 32, 37, 38). Additionally, the integrons harboring MBL determinants are frequently located on plasmids, certainly facilitating their intra- and interspecies spread (32, 33, 37, 43).

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▽ Published ahead of print on 15 October 2007.

TABLE 1. Primers used in this work

Primer	Sequence (5′ to 3′)	PCR product size (bp)	Use
VIM1-F	GTTAAAAGTTATTAGTAGTTTATTG	799	Amplification and sequencing of bla_{VIM-1} and related genes
VIM1-R	CTACTCGGCGACTGAGC		
VIM2-F	ATGTTCAAACTTTTGAGTAAG	801	Amplification and sequencing of bla_{VIM-2} and related genes
VIM2-R	CTACTCAACGACTGAGCG		
VIM-F	AGTGGTGAGTATCCGACAG		Sequencing of bla_{VIM}
VIM-R	ATGAAAGTGCGTGGAGAC		
IMP1-F	ATGAGCAAGTTATCTGTATTC	741	Amplification and sequencing of bla_{IMP-1} and related genes
IMP1-R	TTAGTTGCTTGGTTTTGATGG		
IMP2-F	ATGAAGAAATTATTTGTTTTATG	741	Amplification and sequencing of bla_{IMP-2} and related genes
IMP2-R	TTAGTTACTTGGCTGTGATG		
INT-F	CTCTCACTAGTGAGGGGC	1,010	Amplification and sequencing of *intI*
INT-R	ATGAAAACCGCCACTGCG		
INT-R-I	CGCAGTGGCGGTTTTCAT	Variable	Amplification and sequencing of gene(s) between *intI* and bla_{VIM-2}
VIM2-F-I	CTTACTCAAAAGTTTGAACAT		
qacE-F	GAAAGGCTGGCTTTTTCTTG	210	Amplification of *qacEΔ1*
qacE-R	ATTATGACGACGCCGAGTC		
qacE-F-I	CAAGAAAAAGCCAGCCTTTC	Variable	Amplification and sequencing of gene(s) between bla_{VIM} and *qacEΔ1*
VIM-2-R-I	CGCTCAGTCGTTGAGTAG		
PSE-1F	ATGCTTTTATATAAAATGTGTG	914	Amplification and sequencing of bla_{PSE-1} and related genes
PSE-1R	TCAGCGCGACTGTGATGTA		
OprDF	CGCCGACAAGAAGAACTAGC	1,412	Amplification and sequencing of *oprD*
OprDR	GTCGATTACAGGATCGACAG		
OprDF2	GCCGACCACCGTCAAATCG		Sequencing of *oprD*

A statistically significant increase in imipenem resistance (from 14 to 18%) in *P. aeruginosa* isolates from Spanish hospitals was noted in the second of two sequential multicenter studies performed in 1998 and 2003 (2, 44). This work therefore aimed at analyzing the molecular epidemiology and mechanisms leading to the increasing incidence of carbapenem resistance.

MATERIALS AND METHODS

Bacterial strains. A total of 1,250 nonduplicated *P. aeruginosa* isolates were collected from 127 Spanish hospitals during one week in November 2003 as part of the second national study on the evolution of antimicrobial resistance in *P. aeruginosa* in Spain. The general antimicrobial susceptibility results for this collection of strains have recently been published (44). The present work focused on the characterization of the 236 isolates (18.9%) that were resistant to imipenem (MIC of ≥8 μg/ml) and/or meropenem (MIC of ≥8 μg/ml).

Susceptibility testing. Antimicrobial susceptibility data for the whole (1,250-isolate) collection were available from the previous study in which the MICs of imipenem, meropenem, ticarcillin, piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, ciprofloxacin, ofloxacin, gentamicin, tobramycin, and amikacin were determined using the Neg Combo 1S panels (MicroScan; Baxter Diagnostics, Inc., West Sacramento, CA). Additionally, for particular strains or derivatives, MICs of the same antibiotics were also determined by Etest according to the recommendations of the manufacturer (AB Biodisk, Solna, Sweden). Breakpoints were applied according to Clinical and Laboratory Standards Institute (CLSI) recommendations (5).

Molecular strain typing. The epidemiological relatedness of the strains was studied by pulsed-field gel electrophoresis (PFGE). Bacterial DNA embedded in agarose plugs prepared as described previously (14) was digested with SpeI. DNA separation was performed in a contour-clamped homogeneous electric field DRIII apparatus (Bio-Rad, La Jolla, CA) under the following conditions: 6 V/cm^2 for 26 h with pulse times of 5 to 40 s. DNA macrorestriction patterns were interpreted according to the criteria established by Tenover et al. (47). One isolate per clone and per hospital was randomly selected for further studies.

Detection and genetic characterization of class B carbapenemases. The MBL Etest strips (AB Biodisk, Solna, Sweden), containing imipenem and imipenem-EDTA, were used as the screening technique for the detection of class B carbapenemases. As recommended by the manufacturer, the test was considered positive when the imipenem MICs decreased in more than three twofold dilutions in the presence of EDTA. The presence of MBL-encoding genes was explored by PCR amplification using primers (Table 1) specific for bla_{IMP-1}, bla_{IMP-2}, bla_{VIM-1}, and bla_{VIM-2} or closely related genes, followed by DNA sequencing. Sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). Resulting sequences were then compared with those available in GenBank (www.ncbi.nih.gov/BLAST).

Characterization of genetic elements harboring class B carbapenemases. The possible location of MBL-encoding genes in self-transferable plasmids was evaluated through conjugation assays using either a rifampin-resistant mutant of *P. aeruginosa* PAO1 or a rifampin-resistant mutant of *Escherichia coli* HB101 as the recipient. Transconjugants were selected on Luria-Bertani agar plates containing 100 μg of rifampin/ml and 8 μg (*P. aeruginosa*) or 1 μg (*E. coli*) of imipenem/ml. Additionally, the transfer of MBL-producing plasmids was attempted through transformation experiments. For this purpose, plasmid DNA purified with a QIAfilter plasmid midi kit (QIAGEN, Hilden, Germany) was introduced by electroporation into PAO1 as previously described (46). Transformants were selected on Luria-Bertani agar plates with 8 μg of imipenem/ml and checked using MBL Etest strips and PCR amplification with the corresponding MBL gene primers. Finally, MICs of all the antibiotics listed above for the obtained

transformants were determined by Etest. The integrons harboring the MBL-encoding genes were characterized by PCR followed by DNA sequencing using specific primers (Table 1) to amplify *intI1*, *qacEΔ1*, and the DNA region located between *intI1* or *qacEΔ1* and the corresponding MBL-encoding gene.

β-lactamase assays. To quantify the level of AmpC production, the specific β-lactamase activity (nanomoles of nitrocefin hydrolyzed per min and per milligram of protein) was determined spectrophotometrically with crude sonic extracts as previously described (12). Strains were considered to hyperproduce AmpC when their specific β-lactamase activity was at least 10-fold higher than that documented for PAO1. To detect the production of non-class C β-lactamases, the β-lactamase activity was also determined after the incubation of crude extracts in 50 μM cloxacillin (a class C β-lactamase inhibitor) for 15 min as previously described (12). Extracts showing a >90% inhibition in the presence of cloxacillin were interpreted to produce only AmpC as a major contributor to β-lactamase activity. Strains additionally producing non-class C β-lactamases were excluded from the AmpC analysis. The carbapenemase activity was determined as previously described (19). Briefly, the rate of hydrolysis of imipenem {100 μM solution in 30 mM ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid] buffer, pH 7.0} by crude cell extracts obtained by sonication and resuspension in ACES buffer was measured spectrophotometrically at 299 nm. Hydrolytic activity was also measured after the incubation of the extracts in 2 mM EDTA for 15 min. The VIM-2-producing COL-1 strain (37) and PA2A8, hyperproducing the chromosomally encoded AmpC cephalosporinase (12), were used as positive and negative controls, respectively. For strains producing non-class C β-lactamases and not showing carbapenemase activity, the presence of PSE-1 (CARB-2) or related enzymes, frequently present in *P. aeruginosa* (11), was explored by PCR amplification using the primers described in Table 1.

PCR amplification and sequencing of *oprD*. PCR amplification of *oprD* was performed with whole-DNA extracts from 10 randomly selected imipenem-resistant clones by using the primers described in Table 1 and a DNeasy tissue kit (QIAGEN, Hilden, Germany). In each case, two independent PCR products were fully sequenced as described above, and the resulting sequences were compared with that of the reference strain PAO1.

Quantification of the expression of efflux pumps. Ten randomly selected imipenem- and meropenem-resistant clones and five additional clones resistant only to imipenem were studied. The relative level of *mexB* mRNA was quantified by real-time PCR by following a previously described protocol (12) modified from that of Oh et al. (28). Strains were considered to be MexAB-OprM hyperproducers if the relative expression of *mexB* was at least threefold higher than that by PAO1.

Cloning and characterization of AAC(6′)-32. Primers INT-R-I and VIM2-F-I (Table 1) were used to amplify *aac(6′)-32* from plasmid DNA of *P. aeruginosa* strain GY3. PCR products were ligated to the pGEM-T plasmid to obtain pGTAAC-32, and the *E. coli* XL1-Blue strain made competent by $CaCl_2$ was transformed with pGTAAC-32. Transformants were selected on MacConkey agar plates with 50 μg of ampicillin/ml and were checked by PCR amplification. The cloned *aac(6′)-32* gene was again sequenced to ascertain the absence of mutations produced during PCR amplification. The spectrum of aminoglycosides affected by AAC(6′)-32 was assessed through the determination of the MICs (by Etest) of gentamicin, tobramycin, and amikacin for selected transformants.

Statistical analysis. Categorical variables were compared using Fisher's exact test. A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The nucleotide sequence for the VIM-2-producing integron from strain GY3 has been deposited in the GenBank database under the accession number EF614235. The nucleotide sequence for the *oprD* gene interrupted by an insertion sequence (IS)-like element from strain Def1 has been deposited in the GenBank database under the accession number EF522364.

RESULTS AND DISCUSSION

Epidemiology of carbapenem-resistant isolates in Spanish hospitals. The 236 isolates (18.9% of all *P. aeruginosa* isolates) resistant to imipenem and/or meropenem collected as part of the second national study on the evolution of antimicrobial resistance in *P. aeruginosa* in Spain (44) were studied. These isolates were more frequently isolated from patients with hospital acquired infections than carbapenem-susceptible isolates (58 versus 30%; $P < 0.001$) and were particularly more frequent among intensive care unit patients (36 versus 11%; $P < 0.001$). Remarkably, as shown in Table 2, carbapenem-resistant isolates were also found to be significantly ($P < 0.001$) more frequently resistant to all β-lactams and non-β-lactam antibiotics than carbapenem-susceptible isolates. Furthermore, close to half of the carbapenem-resistant strains met the criteria commonly used for the definition of multidrug resistance (27), and 4% of them were resistant to all antibiotics tested (Table 2).

TABLE 2. Coresistance of carbapenem-resistant isolates to β-lactam and non-β-lactam antibiotics

Antibiotic or resistance phenotype	% of resistant isolates among:		Statistical significance (P)
	Carbapenem-susceptible isolates (n = 1,014)[c]	Carbapenem-resistant isolates (n = 236)[c]	
Ticarcillin	11	34	<0.001
Piperacillin	9	28	<0.001
Piperacillin-tazobactam	6	24	<0.001
Ceftazidime	11	46	<0.001
Cefepime	14	52	<0.001
Aztreonam	19	53	<0.001
Gentamicin	26	59	<0.001
Amikacin	9	19	<0.001
Tobramycin	9	44	<0.001
Ciprofloxacin	25	53	<0.001
Ofloxacin	32	68	<0.001
Imipenem		95[d]	
Meropenem		68	
MDR[a]		46	
Panresistant[b]		4	

[a] MDR isolates are resistant to at least three of the following four antibiotics: ceftazidime, imipenem, tobramycin, and ciprofloxacin (27).
[b] Panresistant, resistant to all antibiotics tested.
[c] The percentages of resistant isolates shown include isolates in the intermediate and resistant CLSI categories (5).
[d] The remaining 5% of strains were imipenem susceptible, and the MIC of meropenem for these strains was 8 μg/ml (CLSI intermediate category).

The molecular epidemiology studies, through PFGE, revealed remarkable clonal diversity, since 165 different clones were identified among the 236 isolates. The extent of clonal dissemination within hospitals was estimated by determining the ratio of infected patients to clones (the clonal dissemination index [CDI]). Most hospitals presented CDIs that did not significantly exceed 1 (which indicates that each patient was infected by a different clone), but some of the hospitals presented higher values, up to 4 (4 infected patients per clone), denoting the occurrence of epidemic or endemic infections during the study period. Indeed, an endemic carbapenem-resistant *P. aeruginosa* clone in a Spanish hospital was recently noted (34). The interhospital transmission of carbapenem-resistant strains was apparently limited, although 6 of the 165 clones were detected in *P. aeruginosa* isolates from more than one hospital. Namely, four clones were detected in two hospitals and two clones were detected in three hospitals. The interhospital spread of carbapenem-resistant *P. aeruginosa* strains has been recognized as a major public health problem in other geographic areas (17), and therefore, active surveillance is needed to detect and prevent the dissemination of these epidemic clones.

Prevalence and nature of transferable carbapenem resistance. The MBL Etest strips were used as the screening tech-

TABLE 3. MICs for the VIM-2-producing *P. aeruginosa* GY3 clinical strain, the corresponding PAO1 transformant harboring plasmid pV2GY3 with bla_{VIM-2} and *aac(6′)-32*, and the *E. coli* XL1-Blue strain harboring plasmid pGTAAC-32 with the cloned *aac(6′)-32*

Strain	MIC (μg/ml) of[a]:											
	PIP	PIP-TZ	TIC	AZT	CAZ	FEP	MER	IMP	CIP	TOB	GEN	AMK
P. aeruginosa												
GY3	16	8	>256	2	16	16	16	>32	>32	>256	32	64
PAO1(pV2GY3)	16	16	>256	2	24	16	16	>32	0.125	64	16	48
PAO1	2	1.5	12	2	1.5	1.5	0.38	2	0.125	0.75	2	3
E. coli												
XL1-Blue										0.38	0.19	0.75
XL1-Blue(pGTAAC-32)										6	0.5	1.5

[a] MICs of piperacillin (PIP), piperacillin-tazobactam (PIP-TZ), ticarcillin (TIC), aztreonam (AZT), ceftazidime (CAZ), cefepime (FEP), meropenem (MER), imipenem (IMP), ciprofloxacin (CIP), tobramycin (TOB), gentamicin (GEN), and amikacin (AMK) were determined by Etest.

nique for the detection of MBL-producing strains, and only 1 of the 236 isolates yielded a positive result. Furthermore, this strain was the only one showing carbapenemase activity when the rates of imipenem hydrolysis were measured spectrophotometrically. As shown in Table 3, this single MBL-producing strain was additionally resistant to all the aminoglycosides tested (gentamicin, tobramycin, and amikacin) and showed high-level resistance (MICs of >32 μg/ml) to ciprofloxacin. PCR followed by DNA sequencing revealed the presence of bla_{VIM-2} in this single strain. A further interesting finding was revealed in the analysis of the PFGE patterns: the single MBL-producing isolate belonged to one of the few epidemic clones that were detected in more than one hospital. Namely, *P. aeruginosa* isolates with identical PFGE patterns were isolated in two additional hospitals in distant geographic areas, although none of those isolates produced the MBL. Indeed, genetic capitalism predicts that the most successful clones are also more likely than other clones to acquire resistance determinants by chance and, as a consequence of the antibiotic pressure in the hospital environment, to be selected and further amplified, leading to epidemics of MDR strains (1).

The cloxacillin inhibition test indicated the presence of non-class C enzymes in seven additional clones, but PCR followed by DNA sequencing revealed the presence of PSE-1 or its close relative PSE-4 in all of these clones. These enzymes are acquired carbecillinases frequently noted in *P. aeruginosa* but certainly do not show carbapenemase activity (3, 4, 11).

Several attempts to transfer the VIM-2-encoding gene from the single MBL-producing strain by conjugation consistently failed, but on the other hand, the gene was successfully transferred by electroporation into PAO1, demonstrating its plasmid location. As shown in Table 3, resistance to aminoglycosides (gentamicin, tobramycin, and amikacin) was cotransferred with the MBL gene, suggesting the codification of both resistance determinants in a plasmid-borne class 1 integron, as described previously for other MBL-producing strains (19, 26, 32, 37, 38). Indeed, the class 1 integron harboring bla_{VIM-2} was characterized by PCR and sequencing, which revealed a highly unusual integron structure, shown in Fig. 1. Particularly noteworthy, the integrase-encoding *intI1* gene was found to be disrupted by a 1.1-kb IS-like genetic element encoding a 370-amino-acid protein 56% identical to a putative transposase recently detected in a *Klebsiella pneumoniae* isolate, in which the transposase was encoded just upstream of the gene encoding the class A carbapenemase KPC-2 (49). This IS-like element contained putative inverted repeats of 20 bp (with three mismatches) and caused a 6-bp duplication of the insertion site (nucleotide 542 of *intI1*). Also interesting, a previously undescribed aminoglycoside acetyltransferase-encoding gene [designated *aac(6′)-32*] preceded bla_{VIM-2}. AAC(6′)-32 showed 80% identity to AAC(6′)-Ib′ and the recently described AAC(6′)-31 (Fig. 2) (16, 26). When cloned, the AAC(6′)-32 gene conferred resistance to tobramycin and reduced susceptibility to gentamicin and amikacin, and therefore, it is most likely to be mainly responsible for the aminoglycoside resistance pattern observed in the MBL-producing strain (Table 3). Finally, a gene encoding a putative 347-amino-acid transposase was found to be

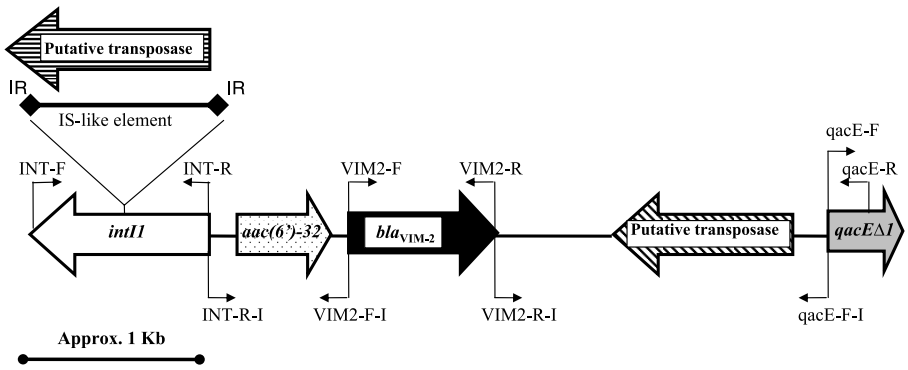

FIG. 1. Structure of the bla_{VIM-2} integron from plasmid pV2GY3 of *P. aeruginosa* strain GY3. The locations of the primers used for PCR amplification are also shown. IR, inverted repeat; approx., approximately.

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AAC(6')-31      ------------MTEHDLPMLHDWLNRPHIVEWWGGEETRPTLAEVLEQYLPSALAKESV 48
AAC(6')-Ib'     ------------MTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESV 48
AAC(6')-32      MSPSKTPVTLRLMTERDLPMLHAWLNRPHIVEWWGGEEERPTLHEVVKHYLPRVLAEEAV 60
                            ***:**.**: ****.********** **** :* ::*** .**:*:*

AAC(6')-31      TPYIAMLDEEPIGYAQSYIALGSGDGWWEDETDPGVRGIDQSLANPSQLGKGLGTKLVCA 108
AAC(6')-Ib'     TPYIAMLNGEPIGYAQSYVALGSGDGWWEEETDPGVRGIDQSLANASQLGKGLGTKLVRA 108
AAC(6')-32      TPYIAMLGDEPIGYAQSYVALGSGDGWWEDETDPGVRGIDQFLSNHTQLNQGLGTKLVQA 120
                *******. *********:**********:********** *:* :**.:******* *

AAC(6')-31      LVEMLFKDAEVTKIQTDPSPNNLRAIRCYEKAGFVAQRTINTPDGPAVYMVQTRQAFEQA 168
AAC(6')-Ib'     LVELLFNDPEVTKIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERT 168
AAC(6')-32      LVELLFSDPTVTKIQTDPAPNNHRAIRCYEKAGFVQQNVITTPDGPAVYMVQTRQAFERV 180
                ***:**.*. ********:*.* ***********  * .:.******************:.

AAC(6')-31      RSAV 172
AAC(6')-Ib'     RSDA 172
AAC(6')-32      RSAA 184
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FIG. 2. Clustal W (version 1.83) multiple-sequence alignment of AAC(6′)-32, AAC(6′)-31 (26), and AAC(6′)-Ib′ (16). Dashes represent amino acid residues that are lacking in the indicated sequence relative to the sequence of AAC(6′)-32; asterisks indicate identical residues, colons indicate conserved substitutions, and dots indicate semiconserved substitutions.

located between bla_{VIM-2} and *qacEΔ1* (Fig. 1). Interestingly, this transposase showed the highest degree of similarity (98% identity) to another putative transposase also corresponding to a bla_{VIM-2}-encoding integron, this one detected in a strain from Thailand (GenBank accession number DQ302723).

Carbapenem resistance mechanisms resulting from chromosomal mutations. The involvement of the classical mutational mechanisms in the carbapenem resistance of the *P. aeruginosa* strains from the Spanish hospitals was also explored. For this purpose, *oprD* genes from 10 randomly selected imipenem-resistant clones were PCR amplified and fully sequenced. As shown in Table 4, 9 of the 10 clones indeed contained inactivating mutations in *oprD*. The most frequent causes of *oprD* mutational inactivation were frameshift mutations produced by 1-bp insertions or deletions and point mutations leading to the creation of premature stop codons, each occurring in three clones (Table 4). These two types of mutations have indeed been found to be the major mechanisms leading to OprD inactivation in other collections, such as that characterized by Pirnay et al. (36). Remarkably, the specific mutations found in two of the Spanish clones, LP2 and SCA1a (Table 4), were previously detected in strains from Portugal and the United States, respectively, as reported in the above-mentioned work. Whether this unexpected coincidence is the consequence of chance, the presence of mutational hot spots, the frequent intraspecies recombinational exchange of *oprD* sequences (36), or the international dissemination of *P. aeruginosa* strains remains to be elucidated. In two additional clones, the inactivation of OprD was driven by a partial deletion of the coding sequence. Finally, in the remaining clone, the inactivation of OprD was caused by the interruption of the coding sequence by a 1.3-kb IS element. This IS encoded a 361-amino-acid transposase 100% identical to that encoded by a sequence recently noted in the chromosome of the *Pseudomonas stutzeri* strain A1501 (GenBank accession number ABP79879). The inactivation of OprD by an IS was also detected in five isolates from a previous study (50), showing the relevance of these elements as a mutational mechanism leading to the inactivation of porins, as also noted for other bacterial species such as *K. pneumoniae* (9).

While OprD inactivation alone is known to result in clinical imipenem resistance (i.e., the resulting MICs for the strain surpass the established resistance breakpoints), the mutational mechanisms leading to clinical meropenem resistance seem to be more complex and are thought to lie in the acquisition of additional mutations (beyond OprD inactivation), such as

TABLE 4. Inactivating mutations in *oprD* in imipenem-resistant clones

Type of inactivating mutation	Clone	Description of mutation(s)[a]
Frameshift mutation produced by 1-bp insertion or deletion	LP2	Deletion of 1 bp (G) at GGGGG repeat (nt 631–635)
	CAS2	Deletion of 1 bp (C) at CC repeat (nt 475 and 476)
	VDS3	Insertion of 1 bp (G) at GGGG repeat (nt 413–416)
Premature stop codon	SCA1a	TG**G**→TG**A** at nt 831
	ALB1	TG**G**→TG**A** at nt 195
	DRP2	TA**T**→TA**A** at nt 219
Partial deletion of the coding sequence	LC4	13-bp deletion beginning at nt 891
	RYC1	25-bp deletion beginning at nt 552
Interruption of the coding sequence by IS	DEF1	1,337-bp IS at nt 1048; encodes a 361-aa putative IS*4* type transposase
None	UNC1	Several nonunique polymorphisms in OprD: T103S, K115T, F170L, E185Q, P186G, V189T, R310E, A315G, and G425A

[a] Nucleotide (nt) and amino acid (aa) numbers are according to the published *oprD* sequence of PAO1. Boldface indicates mutated nucleotides.

those leading to the hyperproduction of AmpC or the hyperexpression of the efflux pump MexAB-OprM (7, 42). The association of AmpC hyperproduction with meropenem resistance was therefore also explored in this work. Notably, 51.3% of the carbapenem-resistant clones evaluated (all the clones detected except those producing non-class C β-lactamases) were found to hyperproduce AmpC. Furthermore, a statistically significant ($P = 0.01$) association with meropenem resistance was found: only 15.6% of the AmpC-hyperproducing clones were susceptible to meropenem, in contrast to 38.9% of the clones showing normal levels of the β-lactamase. Despite the clear association, these results show nevertheless that AmpC hyperproduction is neither sufficient nor necessary for meropenem clinical resistance. The involvement of MexAB-OprM was also explored through the analysis of 10 imipenem- and meropenem-resistant clones and 5 clones resistant to imipenem only. Similar to the results for AmpC, only a partial association of meropenem resistance with MexAB-OprM hyperexpression was obtained: none of the 5 susceptible clones hyperexpressed the efflux pump, but only 3 of the 10 resistant clones showed *mexB* levels at least threefold higher than those in the reference strain PAO1.

In summary, despite the significant increase in carbapenem resistance in *P. aeruginosa* isolates from Spanish hospitals over the last several years and the significant association of carbapenem resistance with multidrug resistance, the prevalence of MBL-producing strains was still very low (0.4% of carbapenem-resistant isolates) when this study was performed (November 2003). These findings are consistent with the very limited number of reports of MBL-producing strains in Spain so far: the first MBL (VIM-2)-producing strain from Spain was documented in a survey performed between 1996 and 2001 in a hospital in Barcelona (41) and was followed by a single strain described years later, detected in a hospital on the island of Majorca (8). The low prevalence of MBL producers in Spain significantly differs from the high prevalence of MBL-producing epidemic or endemic strains in other Mediterranean countries, such as Italy and Greece (15, 40). Nevertheless, very recent reports suggest that the epidemiological situation may be starting to change in Spain also: a report of the first large outbreak of MBL (also VIM-2)-producing *P. aeruginosa* strains has just been published (35), and other VIM derivatives (VIM-1 and the new enzyme VIM-13) are also emerging (C. Juan et al., submitted for publication). Therefore, active surveillance in the coming years is needed to detect and prevent the dissemination of MDR *P. aeruginosa* epidemic clones, particularly those carrying integron- and plasmid-borne MBL determinants, given their additional capacity for the intra- and interspecies spread of multidrug resistance.

ACKNOWLEDGMENTS

This work was supported by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, through the Spanish Network for Research in Infectious Diseases (REIPI C03/14 and RD06/0008) and by the Govern de les Illes Balears (PROGECIC-4C).

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